Remarks

This Reply is responsive to the Office Action dated August 23, 2005. Entry of the amendments and remarks submitted herein and reconsideration of the claimed subject matter pursuant to 37 C.F.R. 1.116 is respectfully requested.

Status of the Claims

Claims 22, 23, 25, 26, 29, 33 to 37, 39 and 49 were pending and under examination at the time of the Office Action dated August 23, 2005. Claims 22 and 49 have been cancelled. Thus, claims 23, 25, 26, 29, 33 to 37 and 39 remain pending and under examination while claims 30 to 32, 38, and 40 to 46 remain pending but withdrawn from consideration.

Amendments to the Claims

Claim 23 has been amended so that it is now an independent claim. Claim 23 has also been amended to indicate that the polypeptide is isolated. Pending claims 25 and 39 and withdrawn claims 40 to 42 have been amended to be dependent on claim 23. Claim 39 has been amended to indicate that agonist or antagonist activity towards a receptor polypeptide of the invention is identified by measuring inhibition of chemotaxis. Written support for these amendments may be found throughout the specification, for instance, page 3, lines 24 to 26; page 4, lines 6 to 7; and page 7, lines 10 to 11. No prohibited new matter has been added by way of these amendments.

Rejections Under 35 U.S.C. 112 (first paragraph)

Claims 22, 25, 29, 33, 34, 36, 37, 39 and 49 were rejected under 35 U.S.C. 112 (first paragraph) for alleged lack of enablement. According to the Examiner, Applicants have not provided sufficient guidance as to how to make variants of SEQ ID NO: 1 or 14 but which still retain the properties of SEQ ID NO: 1 or 14. Applicants respectfully disagree with the Examiner's assertion. Applicants note, however, that the Examiner acknowledges that the claims are "enabling for polynucleotides encoding a protein of SEQ ID NO: 14 and the portion thereof capable of binding ICYP, *i.e.*, SEQ ID NO: 1" (see Office Action at page 2). Applicants further note that claim 23 was not rejected by the Examiner for lack of enablement. In the sole interest of expediting prosecution, Applicants have cancelled claims 22 and 49 and have amended claim 23

such that it is now an independent claim. Claims 25, 29, 33, 34, 36, 37 and 39 are dependent, directly or indirectly, on claim 23. As amended, the claims are enabled given the Examiner's admission in the August 23, 2005 Office Action. Withdrawal of the rejection of these claims is respectfully requested.

In the Office Action, the Examiner maintains that an assay that screens for functional variants of the instant polypeptides by way of measuring relaxation of depolarized-intestinal smooth muscle or eosinophil inhibition is not provided or known in the art, but the Examiner states that the ability to measure relaxation of depolarized-intestinal smooth muscle or inhibition of eosinophil inhibition is not in dispute (see Office Action, page 5, line 15 to page 6, line 2). As discussed above, claim 22, which was directed to SEQ ID NO: 1 or 14 and variants thereof capable of mediating inhibition of eosinophil chemotaxis, has been deleted for the sole purpose of expediting prosecution. Accordingly, the rejection based on this argument is moot.

Claim 39 has been amended to include the step of measuring inhibition of chemotaxis. The Examiner's previous rejection of claim 22 is not applicable to claim 39 because the claim does not cover assaying variants of SEQ ID NO: 1 or 14 for activity. Written support for this amendment is provided, for example, on page 3, lines 14-15 and on page 7, lines 10-11. Further, support for the evaluation of chemotaxis using various cells such as leukocytes, fibroblasts and the like, as well as eosinophils was well known prior to the filing date of the present application. Applicants have previously noted to the Examiner in the Office Action responses dated July 24, 2003 and June 7, 2005 that methods of assaying eosinophil chemotaxis were known in the art, as evidenced by Sugasawa and Morooka (1992) Recent Advances in Cellular and Molecular Biology 3, 223-27. In addition, Applicants herein include as further support Cunningham *et al.* (1991) Science 251, 1233-1236 and Watanabe *et al.* (1985) Japan J. Pharmacol. 39, 102-104. Cunningham *et al.* teach the use of two assays of cell locomotion to assess chemotaxis of NIH 3T3 fibroblasts, including the use of a chemotaxis chamber device (see page 1234, column 1, line 22 to column 2, line 2 and endnote 12). Watanabe *et al.* teach the use of a chemotaxis chamber device to assay chemotaxis of leukocytes. A skilled artisan could use the methods discussed in Cunningham *et al.* or Watanabe *et al.* to assay chemotaxis.

Claims 22, 25, 29, 33, 34, 36, 37, 39 and 49 were rejected under 35 U.S.C. 112 (first paragraph) for allegedly failing to satisfy the written description requirement. The Office Action contends that the claims inappropriately encompass polynucleotides not described in the

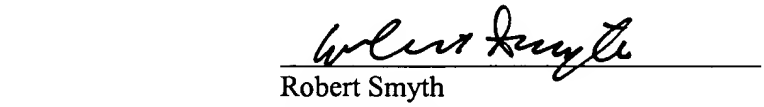
invention, *i.e.*, sequences from other species, mutated sequences, allelic variants or artificial sequences that hybridize to SEQ ID NO: 13 (see Office Action, page 6, lines 10-12). Applicants respectfully disagree with the Office Action's assertion and maintain that sufficient written support is provided in the application for claim 22 without prejudice or disclaimer. However, as stated above, in order to expedite prosecution, Applicants have cancelled claim 22. Claim 23, which the Examiner has not rejected under 35 U.S.C. §112, first paragraph and which the Examiner has acknowledged meets the written description requirement (see Office Action at page 7, line 19 to page 8, line 2), has been amended such that it is now an independent claim. Claims 25, 29, 33, 34, 36, 37 and 39 have been amended, if necessary, to be dependent, directly or indirectly, on claim 23. Accordingly, the Examiner's rejection is now moot.

Reconsideration and withdrawal of the rejections are respectfully requested. This reply is fully responsive to the Office Action dated August 23, 2005. Therefore, a Notice of Allowance is next in order and is respectfully requested.

Except for issue fees payable under 37 C.F.R. 1.18, the commissioner is hereby authorized by this paper to charge any additional fees during the pendency of this application including fees due under 37 C.F.R. 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-0310. This paragraph is intended to be a **Constructive Petition for Extension of Time** in accordance with 37 C.F.R. 1.136(a)(3).

If the Examiner has any further questions relating to this Amendment or to the application in general, he is respectfully requested to contact the undersigned by telephone so that allowance of the present application may be expedited.

Respectfully submitted
Morgan, Lewis & Bockius LLP



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PLC-γ1 was inhibited by a factor present in these cells (possibly profilin). In response to PDGF, tyrosine phosphorylation of overexpressed PLC-γ1 was associated with a higher rate of PI turnover than that observed in untransfected NIH 3T3 cells. However, neither the transient increase in concentration of cytoplasmic Ca^{2+} nor the rate of synthesis of DNA was affected. One interpretation is that activation of PLC-γ1 might control a pathway independent of that leading to the synthesis of DNA (*17*, *18*), such as the pathway responsible for the reorganization of the cytoskeleton. Profilin released from membranes after hydrolysis of PIP_2 by activated PLC-γ1 could participate in such a response.

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12. The level of phosphorylation of PLC-γ1 plateaued (~1 phosphate per PLC-γ1 molecule) after 15 min and remained constant for the next 75 min (*6*). The experimental conditions were selected to optimize the phosphorylation of the tyrosine residue 783 of PLC-γ1, an important substrate for the EGFR and PDGFR (S. G. Rhee, unpublished data). However, other tyrosine residues of PLC-γ1 can also be phosphorylated by the EGFR or RTK in vitro (*6*), and it is likely that our phosphorylated PLC-γ1 preparations were heterogeneous and contained mainly the monophosphorylated PLC-γ1 but also some unphosphorylated and multiphosphorylated enzyme. The PIP_2 hydrolysis assays using the phosphorylated enzyme preparation were all performed within 15 to 35 min after the initiation of the phosphorylation reaction.
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19. Profilin was purified from outdated human platelets [D. A. Kaiser, P. J. Goldschmidt-Clermont, B. A. Levine, T. D. Pollard, *Cell Motil. Cytoskeleton* 14, 251 (1989)] by affinity chromatography on a poly-L-proline agarose column, elution with urea, and renaturation by dialysis against buffer (5 mM tris, pH 7.5, 75 mM KCl, 0.5 mM dithiothreitol, and 0.1 mM NaN_3). PLC-γ1 and PLC-β were purified from bovine brain and stored at −70°C [S. H. Ryu, K. S. Cho, K.-Y. Lee, P.-G. Suh, S. G. Rhee, *J. Biol. Chem.* 262, 12511 (1987)]. EGFR from A-431 cells was prepared by affinity chromatography of cell extracts on wheat germ lectin Sepharose 6 MB (Pharmacia) (*6*).
20. Each PLC assay was performed in a final volume of 100 μl (*8*, *9*). At the end of the incubation, the reaction was stopped by addition of 375 μl of an ice-cold solution of methanol and chloroform (2:1). Further lipid extraction was performed by addition of 125 μl of chloroform and 125 μl of 1 M HCl. The samples were mixed and centrifuged (1000*g* for 5 min at room temperature), and the top 200 μl of the aqueous phase was collected for scintillation counting.
21. All lipids were from Avanti Polar Lipids (Pelham), except PIP_2 (Calbiochem or Boehringer) and phosphatidyl-[2-^{3}H]inositol 4,5-bisphosphate ([^{3}H]PIP_2) (Amersham). Large unilamellar vesicles made of PIP_2 mixed with other phospholipids were prepared by the extrusion technique (*8*, *9*). The concentration of lipid in each mixture was measured by liquid scintillation counting of part of the sample after extrusion.

22. We thank S. H. Snyder and A. L. Hubbard for helpful comments and J. Schlessinger for the RTK preparation. Supported by NIH grant GM-26338 (T.D.P.) and a Fellowship Award of the AHA (P.J.G.-C.).

6 November 1990; accepted 15 January 1991

Enhanced Motility in NIH 3T3 Fibroblasts That Overexpress Gelsolin

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Increasing the content of the actin-binding protein gelsolin in cultured mouse fibroblasts by up to 125 percent by gene transfection proportionally enhanced the rate at which the cells migrated through porous filters toward a gradient of serum and closed a wound made on a confluent monolayer of cells in a tissue culture dish. These results provide direct evidence that gelsolin, which promotes both actin assembly and disassembly in vitro, is an important element in fibroblast locomotion and demonstrate that the manipulation of intracellular machinery can increase cell motility.

EUKARYOTIC CELL LOCOMOTION depends on the assembly and disassembly of actin polymers (*1*), a process regulated by actin-binding proteins (*2*). One such protein implicated in the control of this actin assembly cycle is gelsolin, because it can affect either actin assembly or disassembly and because calcium and polyphosphoinositides, which are intracellular signals, control its interactions with monomeric actin and filamentous actin (F-actin) in vitro. In the presence of micromolar Ca^{2+}, gelsolin promotes the rapid disassembly of F-actin by severing actin filaments, after which it remains tightly bound to the ends of the fragmented filaments (*3*). Membrane polyphosphoinositides, phosphatidylinositol 4-phosphate (PIP) or phosphatidylinositol 4,5,-bis-phosphate (PIP_2), dissociate gelsolin bound to the ends of F-actin, thereby generating free filament ends that act as nuclei for rapid assembly of monomeric actin into filaments (*4*). Actin filaments with ends blocked by gelsolin are therefore potentially key intermediates for eliciting actin assembly at locations where perturbation of cell surface receptors causes appropriate changes in polyphosphoinositide concentrations, conformations, or both. The dissociation of gelsolin-actin complexes precedes net actin assembly in agonist-stimulated cells (*5*), and gelsolin molecules, bound to short actin filaments, have been seen in electron micrographs of cell membranes (*6*), providing indirect evidence that gelsolin-capped actin oligomers may serve as nuclei to promote actin polymerization at the cell's edge.

In a cell stimulated by chemotactic agents, actin disassembly and assembly increase, which accelerates reorganization of the cytoskeleton required for the mechanics of cell movement. If the reversible association of gelsolin with actin regulated by calcium, polyphosphoinositides, and possibly other signals is important for the actin turnover between assembled and disassembled states, then it follows that the gelsolin concentration could be rate-limiting for this cycle. Furthermore, if gelsolin couples the cycle to locomotion through signals generated at the membrane, cell translocation could be quantitatively related to cellular gelsolin content. The observations we report here support this hypothesis.

To examine gelsolin's role in cell motility directly, we permanently transfected NIH 3T3 fibroblasts with the cDNA for human cytoplasmic gelsolin (*7*) in the β-actin promoter-driven expression vector LK444 (*8*), which confers resistance to G418 (*9*), and is designated LKCG. Selection for G418 resistance yielded a mixed population of cells

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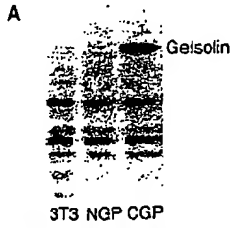
(hereafter referred to as CGP), that constitutively overexpressed varying amounts of cytoplasmic gelsolin. As a control we transfected another set of cells (NGP) with the expression vector alone. Only the CGP cells expressed human gelsolin (Fig. 1A). On the basis of immunoblot and RNA analysis, we estimate that native gelsolin normally comprises about 0.1% of the total cell protein in NIH 3T3 cells and that transfection with LKCG raised this amount by approximately 50% in CGP cells (*10*). The morphologies of parental and transfected NIH 3T3 cells seen by phase-contrast microscopy were indistinguishable, and rhodamine phalloidin staining of the cells did not reveal a difference in the organization of F-actin stress fibers (Fig. 1B) (*11*). Two-dimensional gel electrophoresis of total cell proteins also failed to show any changes in global cell protein expression.

We used two assays of cell locomotion. In one, we measured the migration of cells across a polycarbonate membrane toward a gradient of calf serum (*12*). In the second, we assayed the rate at which a wound scored in a confluent dish of cells (*13*) was closed by the migration of the cells at the edge of the wound by measuring the width of the wound at the same location through an inverted microscope at × 100 magnification with a grid reticule in the eyepiece at timed intervals. In both assays the CGP cells translocated faster than untransfected or control transfected cells (Fig. 2). In the wound closure systems, both control and gelsolin-transfected cells began to enter the wound after a 2-hour lag, after which the rate of migration was constant with time.

To establish if the amount of gelsolin overexpressed could be quantitatively related to motility, we repeated the transfection by LKCG of NIH 3T3 cells, and this time isolated individual clonal lines of resistant cells. Quantitative immunoblot analysis of six clonal lines (designated C1 to C6) showed varying amounts of human gelsolin expressed, from a 25% increase up to a 125% increase above that of the wild-type and control-transfected cells (C1, 25%; C2, 125%; C3, 25%; C4, 90%; C5, 125%; C6, 50% increase, respectively). No morphologic differences were detected in any of the lines by light microscopy; rhodamine phalloidin staining revealed no differences in actin stress-fiber architecture. Growth rates varied slightly among the lines but were not related to the level of gelsolin expression.

All of the clonal lines migrated through a membrane in a chemotactic chamber faster than control (Fig. 3A), and the number of cells migrating correlated with gelsolin expression (Fig. 3B). Wound closure rates for the clonal lines were also increased in comparison with those for control cells (Fig. 3C), and this increase was proportional to gelsolin content (Fig. 3D); gelsolin overexpression enhanced the rate of migration but did not shorten the lag period between wounding and the onset of locomotion (Fig. 3C). In both assays, the increase in translocational motility of the clonal cell lines over control was directly proportional to their increase in gelsolin content (Fig. 3, B and D).

Over- and underexpression of proteins in cells has been a powerful tool for determining the relevance of the particular protein in cellular functions. Although such alterations in expression of cytoskeletal proteins have been used successfully to investigate their function in lower eukaryotes (*14*), the regulation, complexity, and cooperativity of cytoskeletal interactions can confound straightforward interpretations of results, particularly in mammalian cells. For example, microinjection of gelsolin, or of gelsolin proteolytic fragments, or of the gelsolin-related protein villin into cultured cells has had inconsistent effects on the gross mor-



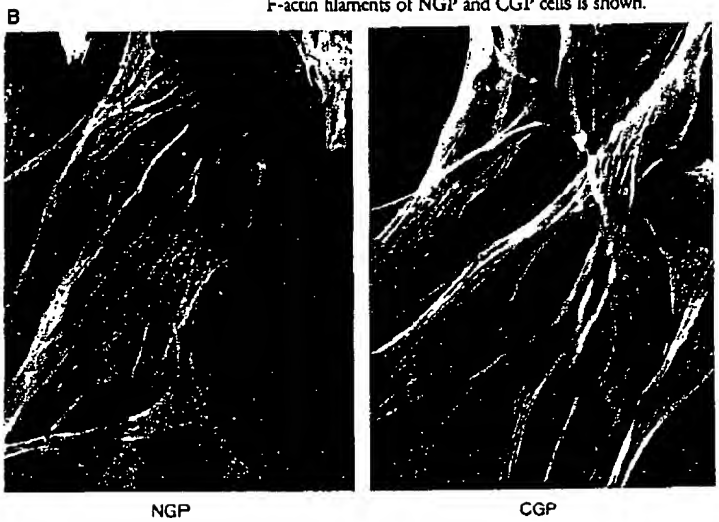


Fig. 1. Expression of human gelsolin in NIH 3T3 cells. (A) Immunoblot showing expression of human gelsolin by transfected NIH 3T3 cells. An autoradiograph of an immunoblot in which a monoclonal antibody specific for human gelsolin and an ^{125}I-labeled secondary antibody are used is shown for the cell lines NIH 3T3, NGP (control transfected cells), and CGP (gelsolin-cDNA–transfected cells). Equal amounts of total cell lysates were loaded in each lane. The lower bands seen are nonspecific and occur inconsistently with this antibody. (B) F-actin stress-fiber architecture of transfected cells. Rhodamine phalloidin staining for F-actin filaments of NGP and CGP cells is shown.

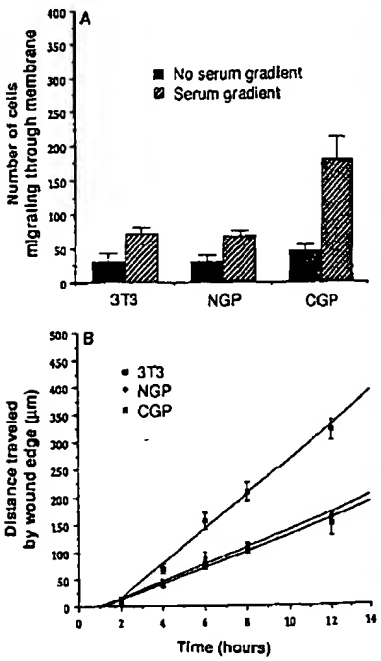


Fig. 2. Migration of gelsolin-transfected NIH 3T3 cells in response to serum. (A) Chemotactic chamber migration (*12*). Calf serum was used as the attractant for 5×10^4 cells. The number of cells traversing the membrane in 2 hours is shown for NIH 3T3, NGP, and CGP cells. (B) Wound closure migration rates. After a lag period, the cells at either edge of the wound began to migrate, and the distance traveled from the wound margin is shown as a function of time for NIH 3T3, NGP, and CGP cells. The slope of this line gives the rate of cell migration and was 15 μm/hour for 3T3 and NGP lines and 31 μm/hour for CGP. Error bars represent standard errors of the mean (SEM) of at least six determinations for both (A) and (B).

phology and actin stress fibers of the cells (*15*). We have found that small increases in gelsolin achieved by transfection do not detectably alter the morphology or gross actin organization of fibroblasts, although such increases appear to enhance the cell's ability to remodel actin in response to signals inducing locomotion. However, we have achieved a much higher level expression of cytoplasmic gelsolin in NIH 3T3 cells in transient transfection experiments and have seen resultant disruption of cell architecture (Fig. 4) (*16*). The deleterious effects of too much gelsolin may explain why the clonal lines we isolated did not express higher levels of gelsolin, because higher gelsolin levels could begin to interfere with normal basal cellular function, leading to a selective growth disadvantage. Thus, there may be a relatively narrow range over which increased gelsolin would lead to enhanced locomotion or other regulated functions.

In summary, modestly increased levels of cytoplasmic gelsolin can enhance the stimulated locomotion of mouse fibroblasts, and this increase in function is proportional to the amount of increased gelsolin expressed, within the range studied. This is the strongest evidence to date of the importance of gelsolin's role in cellular motility and indicates that one could engineer cells with enhanced chemotactic responsiveness. Such cells may be of practical therapeutic use in organ grafting or wound healing.

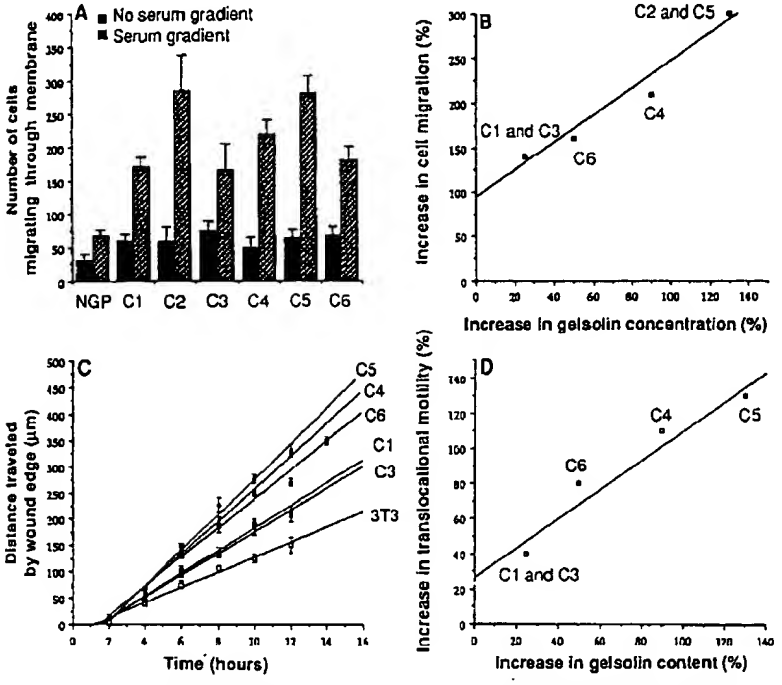


Fig 3. Migration of gelsolin-transfected NIH 3T3 correlates directly with gelsolin expression. (**A**) Chemotactic chamber migration of the six clonal lines. The results shown are the number of cells migrating through the membrane after 2 hours. Error bars are SEM on at least six determinations. (**B**) Relation between the increase in serum-stimulated chamber migration and increase in gelsolin content in six clonal lines. The values shown are relative increases above control values for both migration and gelsolin content. (**C**) Rates of wound closure by five clonal lines. The error bars represent SEM of at least six determinations. The rates of migration were 21 μm/hour for C1 and C3, 27 μm/hour for C6, 32 μm/hour for C4, and 35 μm/hour for C5. Cell line C2 was lost before this assay was completed. (**D**) Relation between migration rate during wound closure and increase in gelsolin content in five clonal lines. The values shown are relative increases above control values for both gelsolin content and rate of migration.

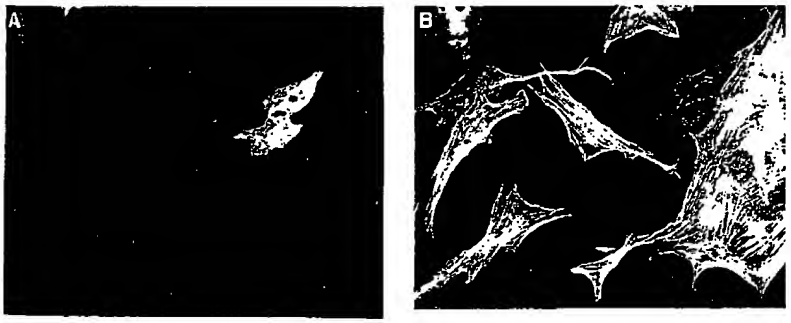


Fig 4. High-level expression of gelsolin in NIH 3T3 cells causes disruption of actin-filament architecture. (**A**) Immunofluorescent staining of transfected cells using the monoclonal antibody to human gelsolin. Two cells stain brightly, indicating gelsolin overexpression. (**B**) Rhodamine phalloidin staining of F-actin filaments in the same cells. A marked reduction of the actin stress-fiber pattern is seen in the two transfected cells in this particular field.

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7. Gelsolin occurs as at least two isoforms, one that is cytoplasmic and the other secreted. A single gene encodes both forms, which are derived from distinct mRNAs generated through the use of alternative transcriptional initiation sites, and many common 3′ exons. The secreted form differs from the cytoplasmic form in that it has a 25–amino acid plasma extension at the NH_2-terminus [D. J. Kwiatkowski *et al.*, *Nature* 323, 455 (1986); _____, R. E. Mehl, H. L. Yin, *J. Cell Biol.* 106, 373 (1988)].
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9. The NIH 3T3 cells were obtained from American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's modified essential medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% bovine serum (Colorado Serum Company, Denver, CO) and with penicillin-streptomycin and gentamycin (Gibco). The selection medium was growth medium with the addition of G418 (Geneticin, Gibco) at a concentration of 1000 μg/ml. LKCG was constructed from vector LK444 by the insertion of a cDNA sequence that encoded cytoplasmic gelsolin, which was constructed from a cDNA for plasma gelsolin by deletion of the signal peptide and other sequence preceding the initiator methionine of cytoplasmic gelsolin. Transfection was performed by calcium phosphate coprecipitation. After a 6-hour, 37°C incubation with the calcium phosphate–DNA mixture, the cells were treated with 10% glycerol for 2 min. After 48 hours in growth media, the plates were changed to selection media.
10. The amount of gelsolin was estimated by quantitative immunoblotting. Dishes were rinsed twice with phosphate buffered saline (PBS), and the cells were then

lysed in an extraction buffer (TEB) (120 mM piperazino-*N*,*N*'-bis[2-ethanesulfonic acid], 40 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, 20 mM EGTA, 4 mM $MgCl_2$, 0.25% deoxycholic acid, 1% Triton X-100) plus protease inhibitors (1 mM benzamidine, 1 mM leupeptin, and 1 mM aprodinin) and scraped off the dish with a rubber policeman. The suspension was vigorously vortexed to disrupt the cytoskeletal components and spun at 10,000*g* for 15 min at 4°C to pellet the nuclei. The protein concentrations of the supernatants were determined by the method of M. Bradford [*Anal. Biochem.* 72, 248 (1976)], and equal amounts of each cell lysate were boiled in denaturing SDS buffer and then separated by electrophoresis on a 5 to 15% SDS-polyacrylamide gel and transferred to Immobilon-P (Millipore, Bedford, MA) by the method of H. Towbin, T. Staehelin, and J. Gordon [*Proc. Natl. Acad. Sci. U.S.A.* 76, 4350 (1979)]. The membrane was incubated at room temperature for 1 hour either with a monoclonal antibody (2 μg/ml) to human gelsolin [D. J. Kwiatkowski, *J. Biol. Chem.* 263, 13857 (1988)] or with a 1:500 dilution of rabbit antiserum to rat gelsolin (*17*), and then with 0.5 μCi/ml of the appropriate ^{125}I-labeled secondary goat immunoglobulin G (IgG) (New England Nuclear, Boston, MA), with washes of 0.2% Tween PBS in between. After further washes, the membrane was exposed to autoradiograph film for up to 12 hours and the film developed. The autoradiograph bands were scanned with a laser densitometer (LKB Pharmacia, Piscataway, NJ) for quantitation of intensity. We used known amounts of mouse gelsolin or human gelsolin to compare band intensity and estimate the amount of native mouse gelsolin and transfected human gelsolin. Expression of native NIH 3T3 gelsolin was not changed in the CGP cells. On the basis of these calculations and estimates of total cell protein and actin content, we determined that actin accounts for 4% of total cell protein and gelsolin 0.1% of total cell protein in NIH 3T3 cells, giving a molar ratio of gelsolin:actin of 1:84, and that in the cell line C5, for example, this ratio was increased to 1:37.

11. F-actin stress-fiber architecture was visualized by rhodamine phalloidin staining. Cells were plated on cover slips, fixed with 0.1% paraformaldehyde and permeabilized in acetone at −20°C, and incubated at room temperature with rhodamine phalloidin (50 U/ml) (Polysciences). The cells were viewed on a Zeiss Axioplan microscope equipped for epifluorescence. Two-dimensional gel electrophoresis was performed by Kendrick Laboratories, Madison, WI, with 2.0% ampholines, pH 4 to 8.
12. Migration through a membrane in response to a chemoattractant was assayed with the use of a 48-well chamber (Nuclepore, Pleasanton, CA) with a 5-μm polycarbonate filter. Cells were trypsinized, then counted, and 5×10^4 cells per well were loaded in the top wells in media plus or minus serum supplementation. The bottom wells were similarly filled with media with or without calf serum so that the cells were exposed to either a gradient, reverse gradient, or no gradient of serum factors. The chambers were incubated for 2 hours at 37°C; the membranes were removed and stained and then examined with a Zeiss Axiovert microscope with a ×40 objective. The number of cells that had migrated through the membrane was counted for each well.
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16. For this transient expression experiment, the cytoplasmic gelsolin cDNA was inserted in the vector CDM8, and this construct was used to transfect NIH 3T3 cells by the DEAE-dextran method [D. J. Kwiatkowski, P.A. Janmey, H. L. Yin, *J. Cell Biol.* 108, 1717 (1989)]. Cells were prepared for staining 2 days later as described (*11*). After permeabilization, cells were incubated with monoclonal antibody (50 μg/ml) to human gelsolin, and then with fluorescein-conjugated goat anti-mouse IgG (50 μg/ml) (Cappel Labs, Cochranville, PA), with washes in PBS with 0.2% gelatin in between. After rinsing, the cells were incubated in rhodamine phalloidin (50 U/ml), rinsed again, and viewed by epifluorescence.
17. We thank K. Weimer for technical help. We thank C. Chaponnier, University of Geneva, for providing the antibody to rat gelsolin. This research was supported by NIH grants HL19429, AI28465, and HL07680. T.P.S. is an American Cancer Society Clinical Research Professor, and D.J.K. is an Established Investigator of the American Heart Association.

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Isolation of Sequences That Span the Fragile X and Identification of a Fragile X–Related CpG Island

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Yeast artificial chromosomes (YACs) were obtained from a 550-kilobase region that contains three probes previously mapped as very close to the locus of the fragile X syndrome. These YACs spanned the fragile site in Xq27.3 as shown by fluorescent in situ hybridization. An internal 200-kilobase segment contained four chromosomal breakpoints generated by induction of fragile X expression. A single CpG island was identified in the cloned region between markers DXS463 and DXS465 that appears methylated in mentally retarded fragile X males, but not in nonexpressing male carriers of the mutation nor in normal males. This CpG island may indicate the presence of a gene involved in the clinical phenotype of the syndrome.

THE FRAGILE X MENTAL RETARDATION syndrome is the most frequent cause of inherited mental retardation (with an incidence of one in 1500 newborn males) (*1*). The diagnosis is based on the presence of a fragile site on the X chromosome, at Xq27.3, induced in vitro by culture conditions affecting deoxynucleotide synthesis (*2*). Partial penetrance is observed in males and females and varies in different sibships, even within the same family (*3*). Many hypotheses have been proposed to account for the unique characteristics of the inheritance of this syndrome. In particular, Laird (*4*) suggested that the fragile site is a region of late replication, resulting from a local inability to reactivate a previously inactive X chromosome, during oogenesis. Using pulsed-field gel electrophoresis (PFGE), we have recently obtained data that support an imprinting mechanism, and found restriction sites that appear methylated in males who express the mutation, but not in normal males whether or not they carry the mutation (*5*). We report here the isolation of a DNA region that spans the fragile site and the identification of a CpG island which appears critically involved in the expression of the syndrome.

We have recently cloned two probes, St677 (DXS463) and Do33 (DXS465), that map within a 3-Mb Not I fragment and that flank breakpoints on the X chromosome purported to be at or very near the fragile X site (*5*, *6*). The distal probe Do33 detects abnormal PFGE patterns in mentally retarded fragile X patients and lies within 120 kb of a region important for the expression of the syndrome (*5*). We have now isolated four yeast artificial chromosomes (YACs) containing St677, Do33, or both probes by direct colony screening (*7*) or by polymerase chain reaction (PCR) screening of YAC pools (*8*). The two larger clones (141H5 and 209G4) were obtained from the total human library of the Centre d'Etude du Polymorphisme Humain (CEPH), and had been derived from a normal male (*9*), and mapped by PFGE after digestion with rare cutting restriction enzymes. We also analyzed the two smaller clones (XY120 and XY530), which were obtained from a Xq24–q28 library that had been derived from the X chromosome of a fragile X

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A New Simple Plastic Chemotaxis Device of the Boyden Chamber Type Utilizing an Immunoassay Plate

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Abstract—A new simple and economical plastic Boyden chamber for in vitro assay of leukocytes chemotaxis was devised by the use of a commercially available immunoassay plate. Zymosan-activated rat serum, formly-methionyl-leucyl-phenylalanine and leukotriene B_4 caused concentration-dependent migration of the leukocytes into the lower chamber. About 50% of the cells loaded in the upper chamber migrated into the lower chamber for 80 min at the optimal concentrations of the three chemoattractants tested.

Leukocyte chemotaxis is an important aspect of host defense mechanisms. The assay method originally described by Boyden (1) for the measurement of leukocyte chemotaxis in vitro had been extensively used in accordance with the original method or with various modifications (2–6). Although the Boyden techniques made powerful contributions to the studies performed in this laboratory on leukocyte chemotaxis (6–10), we felt that it was still an urgent matter to improve the Boyden chamber to make it more economical and reliable as well as more easy to use. The present paper will describe a simplified, highly sensitive and reproducible modification of the Boyden technique for the measurement of leukocyte chemotaxis using a new disposable device made of plastic.

A commercially available immunoassay plate purchased from Eflab Oy Co., Finland (Titertec, Microtitration equipment, Flat bottom type with 12 holes that were each 7 mm in diameter and 11 mm in depth) was used for the lower chambers of the chemotaxis device. Another plate of the same type, of which the bottom of the holes had been filed off by us, was placed upon the above-described lower chambers to assemble the chemotaxis device (Fig. 1). Between each upper chamber and lower chamber, a polycarbonate filter (11–13 mm in diameter; Nuclepore Corp., Pleasanton, CA, U.S.A.) with pores of 2 μm in diameter was tightly sandwiched with the aid of silicon grease (Toray Silicon, Tokyo, Japan) and rubber bands. Prior to placing the filter on the lower chamber, a test solution (4.2 ml) of chemoattractant samples dissolved in RPMI-1640 medium (Nissui Seiyaku Co., Tokyo, Japan) was applied into the lower chamber, being careful to avoid air bubbles.

In order to collect leukocytes, male rats weighing 350–500 g (Sprague-Dawley strain, specific pathogen free, Charles River Japan Inc., Kanagawa, Japan) were injected intraperitoneally with Ca^{2+}-free Krebs-Ringer bicarbonate solution (120 ml/kg body weight) containing 1% casein (Casein nach

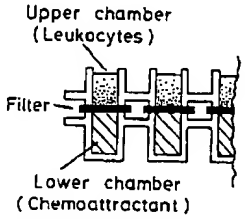

Fig. 1. Newly devised Boyden chamber.

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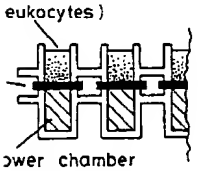

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Hammarsten, Merck). Fourteen hours later, rats were killed by cutting the carotid arteries, and peritoneal fluids were collected and centrifuged at 400×g for 5 min at 4°C, and the cell pellet was washed twice with Gey's balanced salt solution. Cells were finally suspended in the RPMI-1640 medium at a concentration of 1×10^7 cells/ml.

Zymosan-activated serum to be used as a reference cytotaxin was prepared by incubating normal rat serum with 10 mg/ml zymosan (Zymosan A, Sigma Chemical Co., St. Louis, MO, U.S.A.) at 37°C for 30 min then removing zymosan particles under centrifugation, and finally heating at 56°C for 30 min. Normal rat serum treated at 56°C for 30 min was designated as heat-inactivated serum, and used as a control serum with no chemotactic activity.

After the cell suspension (0.3 ml) was placed in the upper chambers, the chemotaxis device was kept at 37°C in a CO_2 incubator with 5% CO_2 and 100% humidity for a suitable period. Then the fluid in the upper chamber was decanted away and remaining fluid and cells in the upper chamber were completely washed away by blowing a jet of water through a pipette. The filter and entire fluid in the lower chamber were then transferred into a test tube and agitated to make a cell suspension. Cells adhering on the inner surface of the lower chamber were detached by blowing a stream of the medium through a capped pipette and pooled with the above cell suspension. The cell number was then counted in a hemocytometer. Migration rate was calculated as follows:

(Number of leukocytes collected from the lower chamber/Number of leukocytes applied in the upper chamber)×100(%).

The leukocyte suspension was applied in the upper chamber charged with 2.5% zymosan-activated serum in the lower chamber, and then the number of leukocytes migrated into the lower chamber was determined after the incubation for 40, 80 and 120 min. The data values obtained were $(1.08\pm0.13)\times10^6$, $(1.60\pm0.09)\times10^6$ and $(1.66\pm0.06)\times10^6$ cells for the respective times, i.e., the number of leukocytes migrated into the lower chamber increased with the passage of time up to 80 min and then

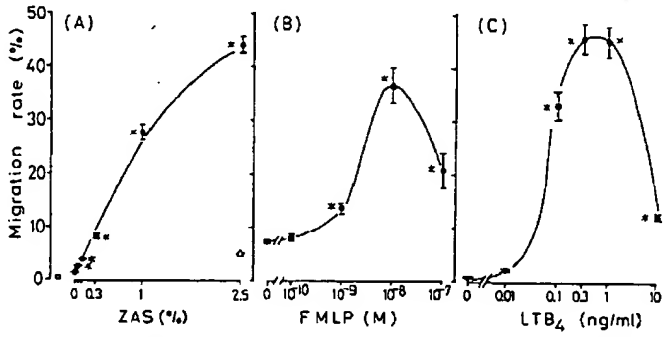

Fig. 2. Leukocyte chemotaxis in response to various chemoattractants. Boyden chambers were incubated for 80 min for the cell migration. Each point represents the mean±S.E.M. of six determinations. Significant difference ($P<0.01$) from respective control is indicated by an asterisk. (A): Leukocyte chemotaxis in response to zymosan-activated serum. Zymosan-activated serum was applied to indicated concentrations in the lower chambers. Heat-inactivated normal rat serum was supplemented to adjust final serum concentration below the filter to 2.5%. A group of the lower chambers contained medium only (□) and another group of the Boyden chambers contained 2.5% of zymosan-activated serum in both the upper and lower chambers (△). (B): Leukocyte chemotaxis in response to FMLP. FMLP dissolved in the medium containing 1% bovine serum albumin was applied in the lower chamber. (C): Leukocyte chemotaxis in response to LTB_4. LTB_4 (kindly supplied by Ono Pharmaceutical Co., Osaka, Japan) dissolved in the medium containing 0.2% bovine serum albumin was applied in the lower chamber.

leveled off. When zymosan-activated serum was applied at various concentrations in the lower chamber, a dose-response relationship in the migration was observed as summarized in Fig. 2 (A). Background levels of the migration rate observed with 2.5% heat-inactivated normal rat serum and the plain RPMI-1640 medium were 1.93±0.27 and 0.80±0.10%, respectively. The migration rate at 0.05% zymosan-activated serum was 2.77±0.07%, i.e., significantly higher than the background levels. When 2.5% zymosan-activated serum was applied in both the upper and lower chambers, the migration rate was 4.9±0.9%, while 2.5% zymosan-activated serum placed only in the lower chamber caused a migration rate of nearly 60%. Therefore, this assay system was evidently capable of detecting the chemotactic activity for 0.05% zymosan-activated serum and distinguishing chemotactic migration of the cells from their random movement.

In addition to zymosan-activated serum, this chemotaxis assay responds sensitively to some other chemotactic substances such as formly-methionyl-leucyl-phenylalanine (FMLP) and leukotriene B_4 (LTB_4) as shown in Fig. 2 (B) and (C). Maximal chemotactic responses to FMLP and LTB_4 were observed at 4.7 ng/ml (1×10^{-8} M) and 0.3 ng/ml (0.9×10^{-9} M), respectively. These data are comparable to those reported by Palmer et al. (11).

Statistical variations of our experimental data were small, and sensitivity was enough to detect a very low level of chemoattractant such as 0.05% zymosan-activated serum, whereas the detection limit of Gallin's method employing ^{51}Cr-labeled granulocytes was reported to be 0.5% zymosan-activated serum (5, 12, 13).

In conclusion, the present modification of the Boyden technique using a disposable plastic device provides a simple, sensitive and reliable assay method for our measurement of leukocyte chemotaxis.

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